

The Maturation of Crystalline Calcium Phosphates in Aqueous Suspensions at Physiologic pH

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Summary. The maturation of calcium phosphate crystals formed by the conversion of spontaneously precipitated amorphous calcium phosphate (ACP) was studied in aqueous media at temperatures ranging from 20° to 37°. Reaction pH was kept at 7.4 with either Hepes buffer or by the pH-stat addition of base. Reaction kinetics were followed by monitoring solution calcium and total phosphate, and, in the pH-stat controlled reaction, by recording the amount of KOH needed to maintain pH. Reaction products were examined chemically and by X-ray diffraction and transmission electron microscopy. The first crystals to form deviated markedly from apatite in morphology, composition, structure, and solubility. They were extremely thin and flaky in appearance, had a low Ca/P molar ratio (1.4), contained an appreciable amount of acid phosphate (16%), and had an exceptionally large *a*-axis (10.5 Å vs. 9.4 Å for apatite). With maturation, the crystals became thicker but smaller in lateral dimensions, more apatitelike in structure and composition, and less soluble. However, this ripening of the crystals was accompanied by unusual inflections in the solution Ca and total PO₄ curves, and, in the case of the pH-stat experiments, in the OH consumption profiles as well. These anomalous post-ACP solution changes suggest that a phase change had taken place during crystal maturation. Although the observed structural and compositional changes are not inconsistent with the perfection of an initially defective apatite, the changes in crystal morphology and the anomalous behavior of the reaction solution may more accurately reflect a conversion of the ACP first into an OCP-like crystalline phase which subsequently hydrolyzes into apatite.

Key words: Amorphous calcium phosphate — Apatite — Calcification — Octacalcium phosphate.

Introduction

To achieve a better understanding of mineral formation in biologic systems, numerous studies have been directed in recent years to the growth of synthetic apatite crystals formed by seeding stable supersaturated solutions (Nancollas and Mohan, 1970; Meyer et al., 1972; Meyer and Nancollas, 1973; Meyer et al., 1974; Nancollas and Tomažič, 1974; Tomažič and Nancollas, 1975; Eanes, 1976). These studies have shown that the precipitation reaction is a complex one involving the formation of nonapatitic calcium phosphate phases during the early stages.

Boskey and Posner (1976) presented evidence suggesting that at the lowest supersaturations which can destabilize spontaneously, i.e., without seeding, apatite nucleated directly from solution without the formation of precursor phases. Subsequent kinetic changes were ascribed to growth of this initial apatite by a crystal ripening process. At higher supersaturations, however, the spontaneous separation of an unstable amorphous calcium phosphate (ACP) preceded the formation of the apatite phase (Eanes et al., 1965; Walton et al., 1967; Termine, 1972). The crystallization of ACP has been described as an autocatalytic conversion process (Eanes and Posner, 1965; Boskey and Posner, 1973) with the first crystals appearing on the surface of the amorphous phase (Eanes et al., 1973). The dependence of this conversion process on the solution environment (Termine et al., 1970), in particular, the role of stabilizing agents such as pyrophosphate and organic phosphonates (Fleisch et al., 1968; Francis, 1969; Robertson, 1973), nucleotides (Blumenthal et al., 1975; Termine and Conn, 1976), and inorganic ions such as magnesium (Eanes and Posner, 1968; Boskey and Posner, 1974) has been extensively studied.

Relatively less attention has been given to the subsequent maturation of the primary crystals formed from the conversion of ACP. Eanes and Posner (1970)

showed that in apatite preparations at pH 10 the crystalline precipitate, remaining after the disappearance of the amorphous precursor, slowly improved in crystal size and perfection with continuous aging of the suspension. This improvement in apatite crystallinity, known as Ostwald ripening (Walton, 1967; Dunning, 1973), occurs commonly in suspensions of sparingly soluble salts. Brečević and Füredi-Milhofer (1972) studied the transformation and crystal ripening of calcium phosphate precipitates in unbuffered media initially adjusted to pH 7.4. Reaction kinetics were followed through changes in solution pH, a procedure, however, which led to acidic solution conditions and the ultimate formation of a precipitate showing mainly the characteristics of octacalcium phosphate (OCP). Comparable kinetic studies carried out under conditions of constant pH were not described. The present work, therefore, was undertaken to study in greater detail at pH 7.4 the nature of the maturational changes occurring in crystalline calcium phosphates formed by conversion of spontaneously precipitated ACP.

Material and Methods

Three different procedures were employed in the preparation of the calcium phosphate suspensions used in this study. In procedure I, stable acidic (pH 4.6) solutions were prepared by the mixing of appropriate volumes of 78 mM $\text{Ca}(\text{NO}_3)_2$ and 48 mM KH_2PO_4 stock solutions with sufficient distilled water to achieve a final volume of 600 ml, a final $\text{Ca} \times \text{P}$ product of $27 (\text{mM})^2$, and Ca/P molar ratios ranging from 1.33 to 1.67. In each preparation the stock phosphate solution was diluted with the distilled water before adding the stock calcium solution. Precipitation was initiated by pipetting rapidly (10 s), while stirring, the amount of 1.77 or 2.3 M KOH needed (approximately 2 ml) to bring the pH of the acidic solution to 7.40. Precipitation occurred immediately with no observable lag time. The pH was then maintained at this value to within ± 0.01 pH units during the entire course of each experiment by means of a pH-stat assembly (Metrohm Combitorator 3D, Brinkmann Instruments, Westbury, N.Y.) with continuous recording of the volume of base needed to keep the pH constant. The same concentration of base used to effect the initial precipitation was employed to control the pH.

In procedure II, a 300 ml solution of 13.35 mM $\text{Ca}(\text{NO}_3)_2$ was added rapidly (10 s), while stirring, to a 300 ml basic solution 8.0 mM in KH_2PO_4 . The amount of KOH used to make alkaline the phosphate solution (pH 11.1) was the same as that needed to initiate precipitation in the corresponding experiment under procedure I. Precipitation took place without any perceptible delay. Following mixing of the reactants, the pH was maintained at 7.40 ± 0.01 by the same pH-stat procedure as described for procedure I.

In procedure III, the calcium phosphate suspension was prepared by mixing together rapidly (10 s) equal volumes of $\text{Ca}(\text{NO}_3)_2$ and Na_2HPO_4 solutions, each buffered at pH 7.4 with 25 mM Hepes (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid)/NaOH. Before mixing, the calcium and total phosphate concentrations were either 7.80 and 4.80 mM, respectively ($\text{Ca}/\text{P} = 1.625$), or both 6.12 mM ($\text{Ca}/\text{P} = 1.0$). These concentrations were sufficient to produce immediate precipitation.

All precipitation reactions were carried out in double-walled cylindrical pyrex vessels under CO_2 -free N_2 . Unless otherwise noted, all solutions were stirred at 240 rpm and the temperature kept constant by circulating water at either 20, 28, or $37 \pm 0.1^\circ$ between the walls of the reaction vessel. All solutions were prepared with reagent grade chemicals and deionized distilled CO_2 free water. The pH of the reaction solution was measured in situ with combination glass electrodes standardized with at least two National Bureau of Standards buffers encompassing the pH range of interest.

Either 5 or 40 ml portions of the reaction mixture were taken at regular intervals during the course of each experiment for chemical and/or X-ray analysis. Samples were withdrawn using plastic syringes and filtered immediately by dispensing through Swinnex assemblies (Millipore Corporation, Bedford, Mass.) equipped with 0.1 or 0.22 μm MF-Millipore filters. Approximately 10 s lapsed between the time a 5 ml sample was withdrawn from the reaction slurry to the time filtering was completed. The filtrates collected from the 5 ml samples were analyzed for solution calcium and total phosphate concentrations. The 40 ml portions were taken primarily for solid analyses with less than 1 min required to withdraw, filter, and freeze the retained residues in liquid N_2 for lyophilization. Duplicate calcium, acid phosphate (HPO_4^{2-}), and total phosphate determinations were done on all solids.

All calciums were determined by atomic absorption spectrophotometry using a model 444A AA analyzer (Beckman Instruments, Fullerton, Ca.). Total phosphate was determined by the differential UV spectrophotometric method of Murphy and Riley (1962). Acid phosphate was measured indirectly by the heat-induced pyrophosphate method of Gee and Dietz (1955). Samples were ignited in vacuo at 550° for 16 h. Following ignition the amount of HPO_4^{2-} pyrolyzed was obtained by determining orthophosphate before and after hydrolysis for 1 h in 1.0 M HClO_4 at 100° .

The standard deviations (SD) of the solution measurements were less than 0.01 mM. For solids, the results of the calcium and total phosphate analyses are given as Ca/P molar ratios (± 0.02 SD). The error in the acid phosphate procedure was $\pm 1.0\%$ of the total PO_4 value.

In the pH-stat controlled experiments, free ionic activities were obtained from the measured concentrations by correcting for ion pairs (Chughtai et al., 1968) and by using activity coefficients estimated by the extended Debye-Hückel equation as proposed by Davies (1962). These activities were used in turn to determine how saturated the solution was throughout the reaction with respect to the following calcium phosphate salts: hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$; HAP), octacalcium phosphate ($\text{Ca}_8\text{H}(\text{PO}_4)_6 \cdot 2.5\text{H}_2\text{O}$; OCP), tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$; TCP), and dicalcium phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$; DCPD). The degree to which the reaction solution was over- or undersaturated with respect to each of the above phases was computed using the following expression:

$$\Delta G = - \frac{RT}{N} \ln \frac{IP}{K_{sp}}$$

where R is the ideal gas constant, T is the absolute temperature, IP is the product of the free ionic activities based on the simplest molecular formula of each phase, N is the number of ions in the ion product, and K_{sp} is the thermodynamic solubility product. The values of K_{sp} used for HAP, OCP, TCP, and DCPD at 37° were respectively: 2.12×10^{-29} (McDowell et al., 1969), 1.06×10^{-48} (Madsen, 1970), 0.22×10^{-29} (Moreno et al., 1970), and 2.19×10^{-7} (Gregory et al., 1970). The above values of K_{sp} refer to well-characterized crystalline calcium phosphates only and, therefore, do not take into account Gibbs-Kelvin effects (Walton, 1967) which

would tend to increase the solubility of these materials when they are in a finely divided heterodisperse state.

X-ray diffraction patterns of the solid phases were obtained either photographically using standard 114.6 mm diameter X-ray powder cameras or digitally using a Siemens electronic diffractometer in automatic step-scanning mode. Copper $K\alpha$ radiation was employed in both procedures.

At regular intervals, fresh specimens of the precipitation reactions were obtained for transmission electron microscopy (TEM) by the drop-drain method of Eanes et al. (1973). Lyophilized specimens were also examined by transferring the dry powders directly to specimen grids. Micrographs were obtained using an AEI-6B electron microscope operated at 60 kV.

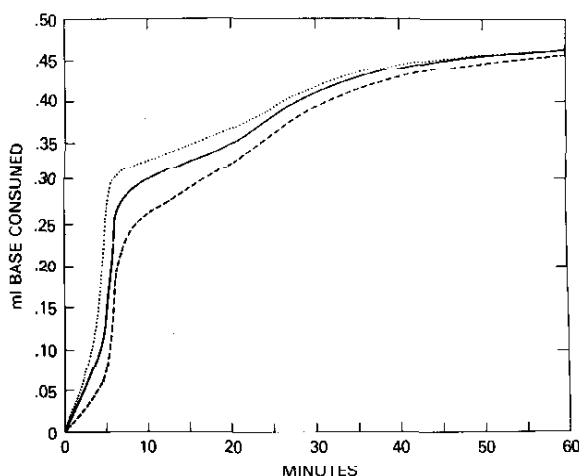


Fig. 1. Time plots of the volume of 2.30 M KOH required to maintain the pH at 7.4 in three (pH-stat controlled) precipitation reactions at 37°. Precipitation was initiated by procedure I from solutions in Ca/P molar ratio of 1.33 (.....), 1.45 (—), or 1.67 (---)

Results

Figure 1 shows the OH-consumption with time for three pH-stat controlled experiments at 37°. In these experiments, precipitation was initiated by procedure I from solutions in which the Ca/P molar ratio was either 1.33, 1.45, or 1.67. The curves show that in each case the rate of addition of base needed to keep the pH constant at 7.4 was moderately fast for the first few minutes, then sharply increased. By 6 min, however, this sharp rise in OH-consumption had begun to taper off into a period of relatively slow but constant consumption. At approximately 20 min another upswing was observed, much less prominent than the first but still well defined. This second upturn was then followed by continually decreasing rates in OH-uptake. Although not shown, this tailing off in the OH-curves continued without further inflections for the remainder of each experiment (up to 16 h).

In a parallel series of experiments where all conditions were the same except samples were regularly removed for analysis, X-ray diffraction and TEM examination of solid reaction products showed that the initial precipitate was ACP, but that by 6 + 1 min the precipitate had fully crystallized with no evidence of any remaining amorphous material. Therefore, the steep rise in the OH-curves seen in Figure 1 was associated with the ACP conversion reaction. Since this reaction has been the object of many earlier investigations and is well documented (see references given in the Introduction), the remaining data presented will, in the main, describe only the postconversion behavior of the reaction system.

The continued increase in OH-consumption following completion of the ACP conversion (Fig. 1) shows that the initial crystalline precipitate formed had undergone further changes. The most interesting feature in the OH-curves associated with these postconversion changes was the renewed upswing commencing at about 14 min after the ACP stage was over. The appearance of this inflection in the OH-consumption was relatively unaffected by the Ca/P molar ratio of the starting reactants with the inflection being only somewhat more noticeable when a ratio of 1.45 was used. In a like manner, doubling the stirring rate to 480 rpm or halving it to 120 rpm did not change the time at which this upward swing in OH-consumption took place. Figure 2 shows that the sequence in which the reactants were mixed to initiate precipitation also had little effect on the postconversion inflection. Beginning the pre-

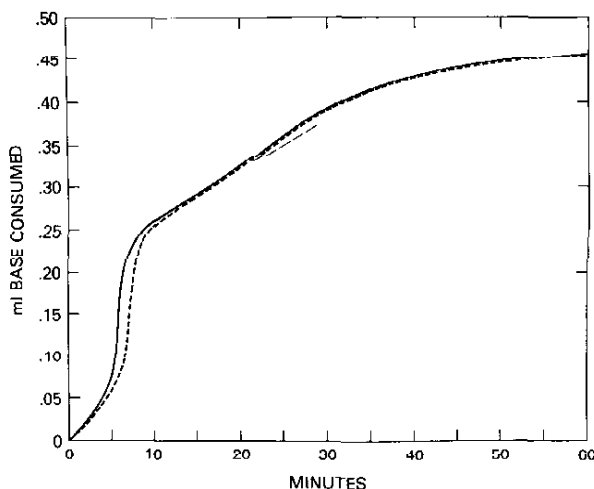


Fig. 2. Time plots of the amount of 2.30 M KOH needed to keep the pH constant at 7.4 in 37° precipitations controlled with a pH stat and initiated by either procedure I (solid line) or procedure II (short-dash line). The starting Ca/P molar ratio in each case was 1.67. A long-dash line is drawn tangential to the base uptake curves at 20 min to delineate the magnitude of the inflection

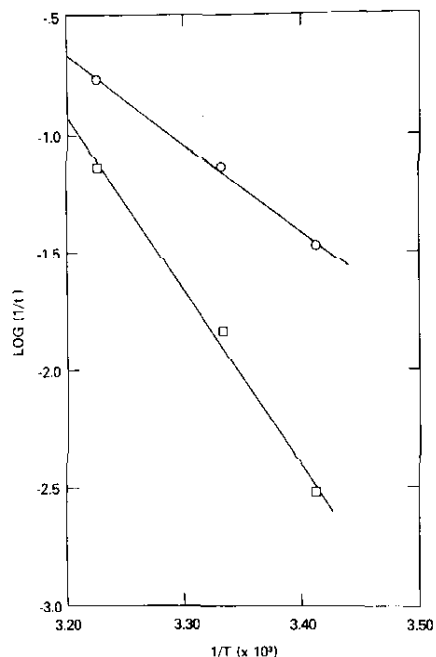


Fig. 3. Arrhenius plots of the effect of temperature on the amorphous/crystalline transition time (circles) and the postconversion inflection time (squares) in precipitations initiated by procedure I and controlled at pH 7.4 with a pH stat. The logarithm of the reciprocal of the time interval (t) preceding each of these two precipitation events is plotted against the reciprocal of the absolute temperature (T)

precipitation reaction under conditions of alkaline rather than acidic solution did not modify the magnitude nor the shape of the postconversion inflection and only slightly delayed its appearance. On the other hand, the data in Figure 3, given in the form of Arrhenius plots with the logarithm of reciprocal time plotted against the inverse of the absolute temperature (Boskey and Posner, 1973), shows that the effect of reaction temperature was considerable. Decreasing the temperature from 37° to 20° lengthened the interval between the end of the conversion and the beginning of the inflection from 14 to 338 min. As Figure 3 also shows, the ACP conversion time had likewise increased with decreasing temperature but at a much slower rate.

Figures 4 and 5 show that anomalous postconversion inflections were also observed in the calcium and total phosphate concentration curves. In par-

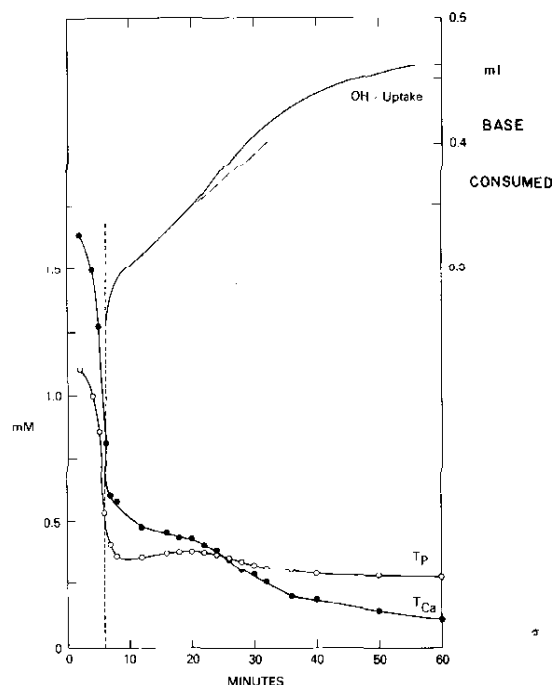


Fig. 4. Concentrations of solution Ca and total PO_4 vs. time are plotted for a (pH-stat controlled) precipitation initiated by procedure I in a solution initially 1.45 in Ca/P molar ratio. Also plotted (OH-uptake) is the volume of 2.30 M KOH needed to keep the pH constant at 7.4 during the postconversion period. A dashed line (---) tangential to this latter curve at the beginning of the inflection delineates more clearly the magnitude and shape of this inflection. The vertical dashed line in this and subsequent figures denotes the separation between the ACP and post-ACP stages of the reaction

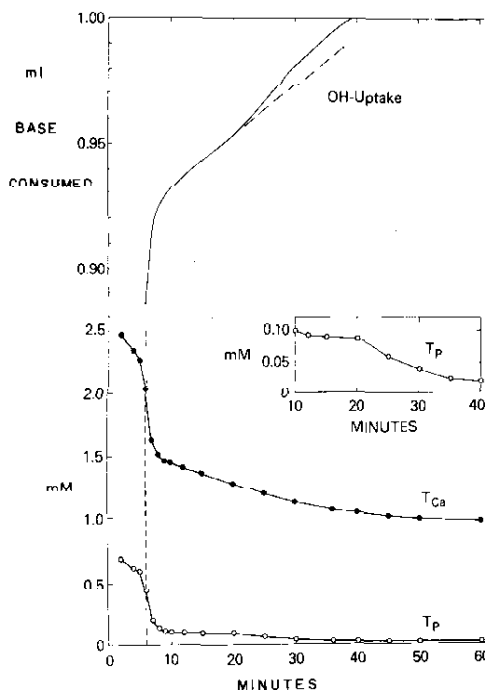


Fig. 5. Plots of the solution Ca and total PO_4 concentrations vs. time in a procedure I precipitation with an initial Ca/P molar ratio of 1.67. Also plotted (OH uptake) is the postconversion part of the curve describing the amount of 1.77 M KOH needed to keep the pH constant at 7.4. The tangential and vertical dashed lines have the same meaning as in Figure 4. Insert shows an expanded plot of the solution PO_4 changes in the region of the postconversion inflection point

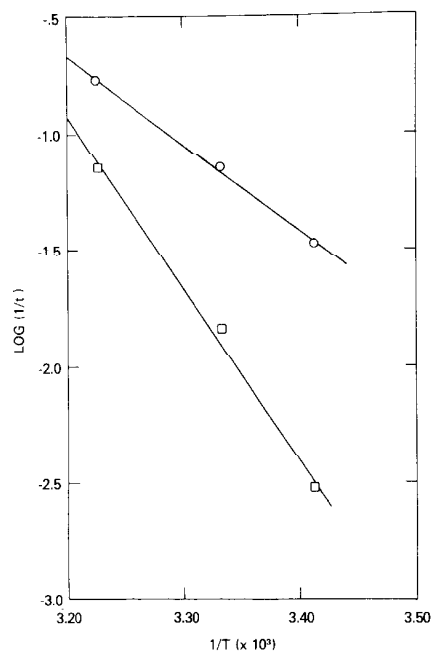


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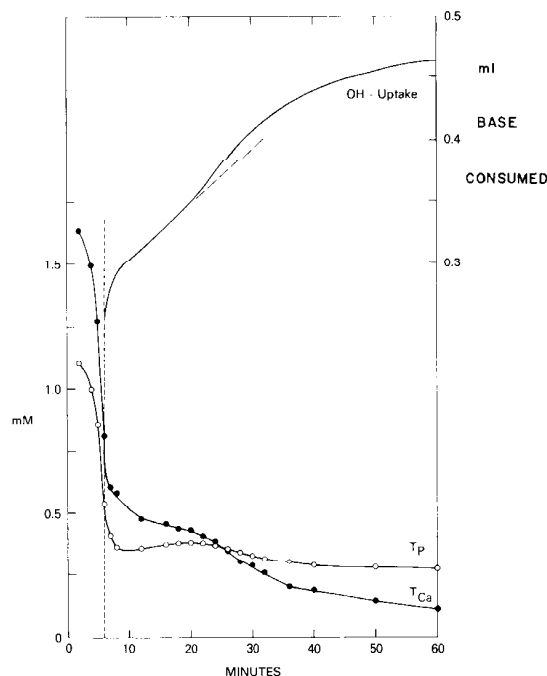


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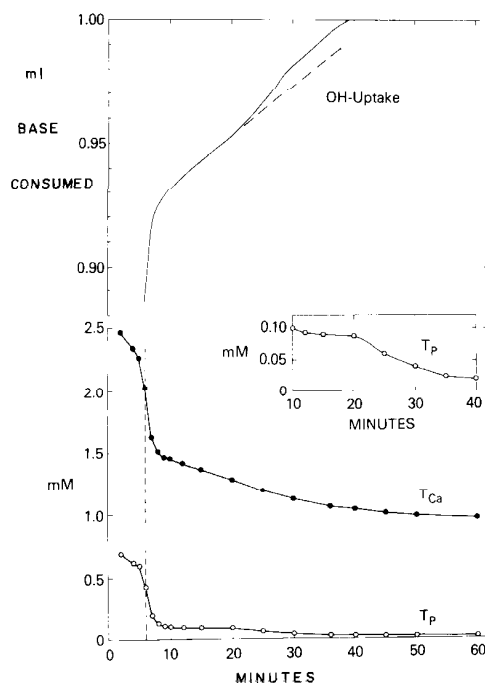


Fig. 5. Plots of the solution Ca and total PO_4 concentrations vs. time in a procedure I precipitation with an initial Ca/P molar ratio of 1.67. Also plotted (OH uptake) is the postconversion part of the curve describing the amount of 1.77 M KOH needed to keep the pH constant at 7.4. The tangential and vertical dashed lines have the same meaning as in Figure 4. Insert shows an expanded plot of the solution PO_4 changes in the region of the postconversion inflection point

ticular, distinct increases in the rate of loss of these ions from solution occurred concurrently with the upswing of the OH-consumption. These accelerations in losses of solution ions were quite marked for phosphate, where they were preceded either by a period of nearly constant concentration (Fig. 5, insert) or, as in the case where the Ca/P ratio of the starting reactants was 1.45 or less, by a period of rising phosphate concentration (Fig. 4). As can be seen from the ΔG plot in Figure 6, this period of secondary increase in solution phosphate reflected only a leveling off and not a reversal in the previously steady decline in precipitate solubility. Apparently the decrease in solution calcium during this period was sufficiently great to offset the rise in solution phosphate.

By giving a clearer picture of the total solution behavior, Figure 6 also shows that the inflections seen in the raw concentration data were signalling an abrupt increase in the rate at which the crystalline precipitate was decreasing in solubility with aging. The total change in solubility resulting from this increase in rate was comparable in magnitude to that seen accompanying the ACP conversion. In addition, the ΔG curves show that even though the precipitate at the beginning

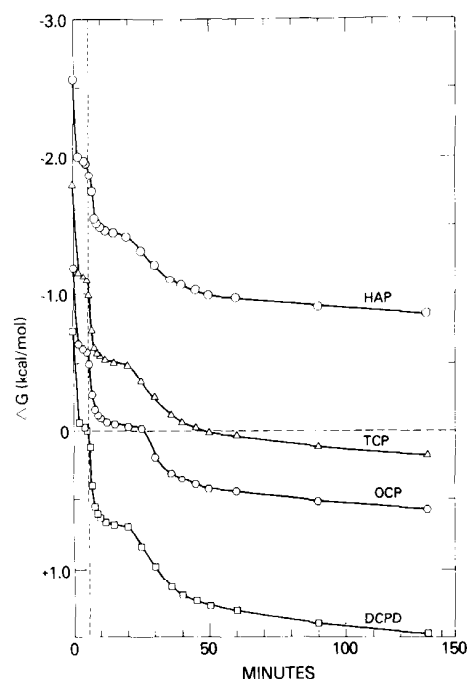


Fig. 6. Time variation in the degree of saturation (ΔG) of the pH-stat controlled solution at 37° with respect to the calcium phosphate phases HAP (○), TCP (△), OCP (◐), and DCPD (◑). The horizontal dashed line at $\Delta G = 0$ is the point at which the solution is saturated with respect to the solid phase. Region above this line represents supersaturation; below this line, undersaturation. The vertical dashed line has the same meaning as in Figure 4

of the postconversion period was more soluble than either OCP, TCP, or HAP, with aging the precipitate only exceeded HAP in solubility. Most interestingly, the ΔG curve for OCP indicates that the precipitate was approaching the solubility of this phase when the inflection in crystal ripening occurred.

Postconversion maturation of the crystalline solids formed in the pH-stat controlled precipitations was characterized chemically by increases in the molar Ca/P ratio (from 1.42 to 1.57) and by concomitant decreases in acid phosphate content (from 14.7% to 0.8%) (Fig. 7). These changes in precipitate chemistry proceeded most rapidly during the first hour of crystal ripening. Therefore, even though they themselves do not display any unusual inflections, they encompassed the period of secondary solution change described in the previous paragraphs.

X-ray examination of the postconversion solids showed that the only phases present exhibited apatite-like diffraction features. However, the interplanar spacing for what would normally be the 100 apatite X-ray reflection was exceptionally large for the earliest-formed crystals (9.11 Å vs. 8.17 Å for stoichiometric hydroxyapatite, equivalent to an expansion in the a -axis from 9.4 Å to 10.5 Å). With solution aging, this d-spacing steadily decreased toward more typical values,

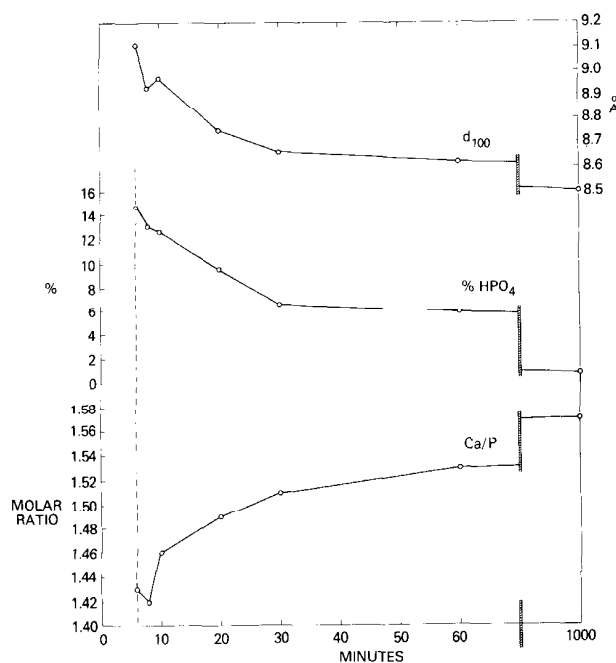


Fig. 7. Time dependency in the 100 d-spacing (Å — Angstroms), % HPO_4 , and molar ratio of Ca/P for crystalline solids isolated during the postconversion stage of a pH-stat controlled experiment at 37°. (Procedure I; reactant ratio of 1.67 for Ca/P)

although at 16.7 h it had not yet fallen below 8.49 Å (Fig. 7). A slight but significant expansion of the 002 d-spacing also occurred with ripening (3.421 to 3.444 Å).

Figure 8 shows the solution calcium and total phosphate changes in a Hepes-buffered experiment at 37° and pH 7.4. The conversion was more protracted than for the pH-stat reaction with the postconversion changes beginning at 25 min. However, the reaction curves shown in this figure exhibited the same general features as described earlier, including the distinctive postconversion inflections in calcium and total phosphate concentrations (beginning at 35 min).

Figure 9 shows that the starting molar ratio of the Hepes-buffered reactants had only a minor effect on the conversion, with completion occurring somewhat sooner when the reaction was carried out in excess solution PO_4 . The reactant ratio appeared to have even less effect on the subsequent solution inflections. Similarly, the Ca/P and % HPO_4^{2-} of the earliest-formed crystals were relatively unaffected by the

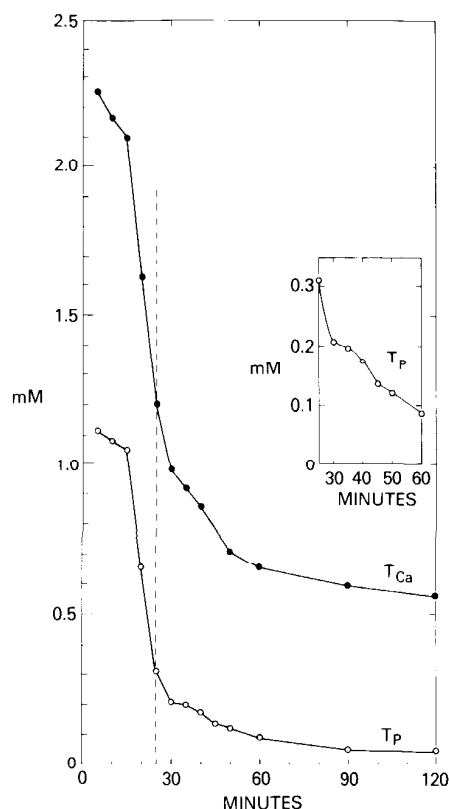


Fig. 8. Concentration changes of Ca and total PO_4 vs. time for a Hepes-buffered precipitation reaction at 37°, pH 7.4, and reactant ratio of 1.625 for Ca/P. Insert is an expanded plot showing the postconversion inflection in solution PO_4 .

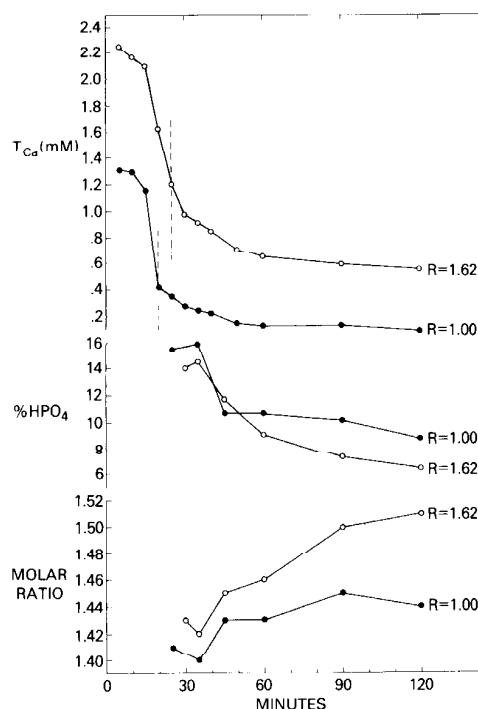
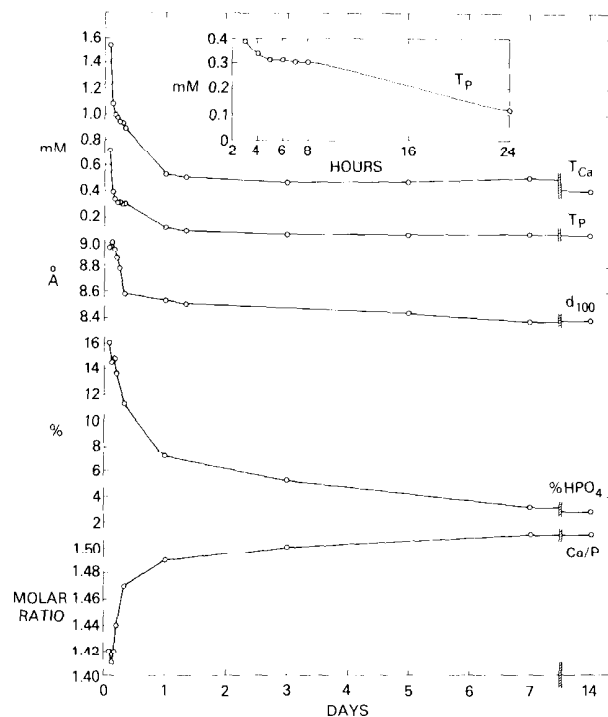


Fig. 9. Changes in solution Ca, % HPO_4 (solid), and molar ratio of Ca/P (solid) vs. time for Hepes-buffered preparations at 37° and for reactant ratios of 1.00 and 1.625 for Ca/P.

reaction ratio. However, the solid data did show a gradual divergence in the rate of crystal ripening, with the crystals bathed in excess solution phosphate maturing less rapidly. The earliest crystals were also comparable in composition to their pH-stat controlled counterparts, a similarity which extended to diffraction features as well, as evidenced by an expanded d_{100} spacing of 8.84 Å which decreased in time to 8.42 Å.

The results of Hepes-buffered experiments conducted at 20° are given in Figure 10. As with the pH-stat experiments, lowering reaction temperature retarded the conversion considerably (from 25 min to 2 h). Postconversion events were similarly affected with inflection in the solution curves not occurring until 8 h into the reaction. As with the 37° experiments, the inflection was most noticeable in the total phosphate solution curve where it was preceded by a distinct leveling off in solution phosphate losses (Fig. 10, insert). Accompanying the major contraction in d_{100} was a less pronounced expansion in d_{002} (3.39 to 3.44 Å).

In transmission electron micrographs of 37° Hepes-buffered precipitates taken immediately after conversion, the freshly formed crystallites were observed clustered into rosettelike aggregates reflecting their



genesis from ACP (Eanes et al., 1973) (Fig. 11A). Individual crystallites were flaky, extremely thin (9–17 Å), and fragile in appearance. Many of the crystallites appeared wrinkled and/or curved rather than smooth and flat. This warped aspect together with poor delineation between crystal boundaries gave the clusters a soft, flowing texture. These features also made it difficult to assess crystal dimensions, but where individual crystallites were sufficiently isolated to be measured they had discernible faces as large as $1.3 \times 10^6 \text{ Å}^2$ in area (with edges as long as 1500 Å).

With solution aging, the clusters became more particulate in appearance (Fig. 11B). Individual crystallites within each cluster were better delineated from their contiguous neighbors with clearly resolved though irregular borders. The crystallites were decidedly thicker (up to 40 Å), imparting a stiff, platelike aspect to their surface features. This increase in crystal thick-

Fig. 10. Postconversion changes in solution Ca and total PO_4 , and in the d_{100} , % HPO_4 , and molar Ca/P of the solids vs. time for a Hepes-buffered preparation at 20°, pH 7.4 and Ca/P (reactant ratio) = 1.625. Insert shows the inflection point in the PO_4 solution profile

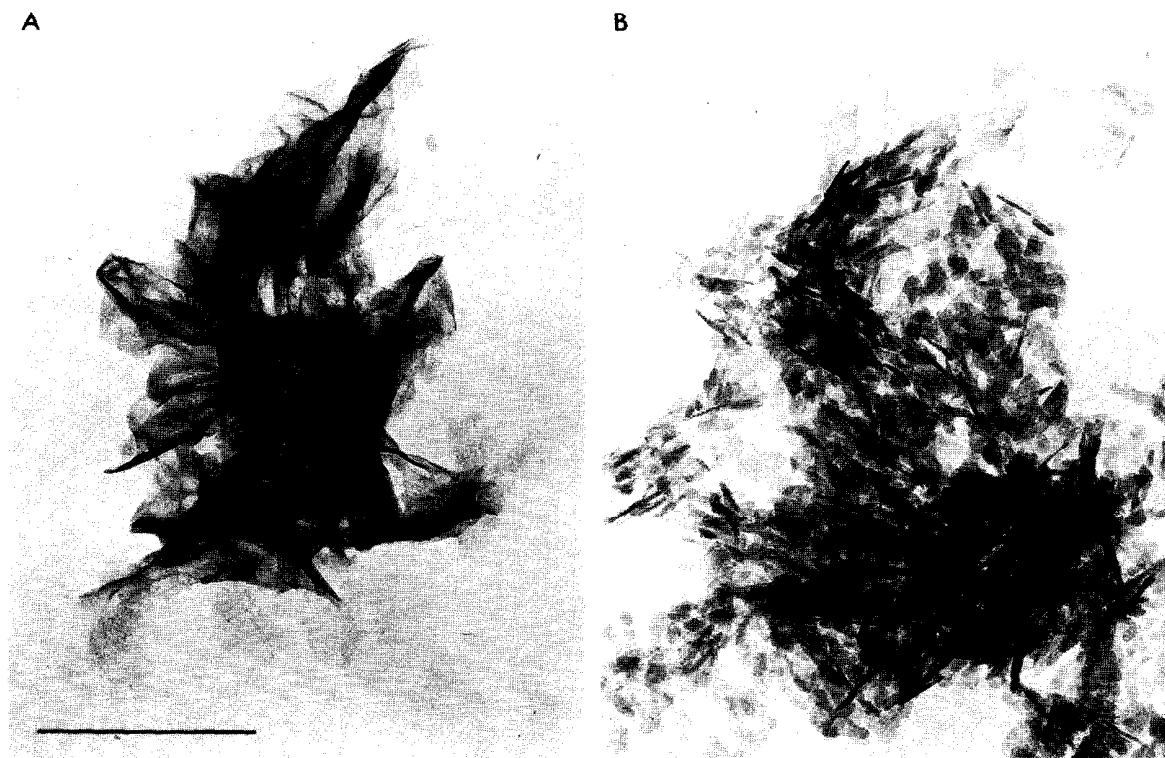


Fig. 11. TEM of crystal clusters obtained from a 37° Hepes-buffered suspension (A) immediately after conversion of the ACP (25 min) and (B) at the end of the experiment (68 h). Length of horizontal bar = 0.25 μm



Fig. 12. TEM of crystalline material taken from a 37° Hepes-buffered suspension shortly after the appearance of the solution inflection point (90 min). Length of horizontal bar = 0.25 μm

ness, however, was not accompanied by corresponding growth in other directions. On the contrary, there was a considerable reduction in the dimensions of the broadest faces (max. < 1100 Å). Face areas greater than $0.4 \times 10^6 \text{ Å}^2$ were not observed.

This changeover into thicker, smaller crystals appeared to occur *in situ*. Clusters harvested shortly after the postconversion inflection point still retained a semblance of their initial form, but an apparent breaking up of the original crystal surfaces had taken place, imparting a coarse, pebbly texture to the clusters (Fig. 12). This deterioration in the appearance of the original clusters was not a result of collisions brought about by solution stirring, as this same effect was observed in samples taken from unstirred preparations. The above-described crystal features were also observed in precipitation reactions which were pH-stat controlled.

Discussion

In the absence of such foreign ions as F and CO_3 , hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) is the thermodynamically stable end product of calcium phosphate precipitations carried out at physiologic pH and temperature. Data obtained in the present study show, however, that although apatitelike in their general structural features, the first crystals to form in

spontaneously precipitating systems deviate markedly from HAP in composition and structural details, as exemplified by low Ca/P molar ratios, high levels of HPO_4^{2-} , and distorted lattice dimensions. These primary crystals, moreover, are unstable and, with concomitant decreases in solubility, steadily change upon solution aging to compositions and structures more nearly resembling HAP. The exponential-like rate at which these chemical and structural changes decrease with time would suggest a maturation behavior similar to that observed in other sparingly soluble inorganic precipitates (known as Ostwald ripening, Walton, 1967; Dunning, 1973).

The presence of well-defined, concurrent inflections in the hydroxide, calcium, and phosphate solution profiles indicates, however, that the postconversion crystallization process is more complicated than can be described by a steady change in the growth and perfection of a highly defective apatite. These inflections could possibly be explained by the hydrolysis of other acidic calcium phosphate phases formed with the ACP at the time of the original precipitation. Evidence against this interpretation, however, is the finding that the inflections were not dependent upon the manner in which the ACP was initially precipitated. Essentially identical results were obtained from ACPs prepared with highly alkaline phosphate solutions, which minimized the possibility of acidic phosphate contaminants forming, as with phosphate solutions initially low in

pH. A more plausible interpretation is that the observed inflections reflect an intrinsic property of the crystal maturation process itself. In particular, they indicate that the maturation occurs in at least two distinct stages. In addition, the effect of temperature on the OH-inflection, as evidenced by the linearity of the Arrhenius plots, suggests that the maturation change connecting these two stages is, like the ACP conversion that precedes it, a single, well-defined kinetic event and is not the result of the superposition of a number of different kinetic processes.

Nancollas and co-workers (Nancollas and Tomažič, 1974; Tomažič and Nancollas, 1975; Tomažič et al., 1976) noticed similar inflections in the growth curves of stable supersaturated calcium phosphate solutions seeded with HAP. These authors concluded that such inflections pointed to an involvement of more than one phase in their seeded precipitations. Evidence that this multiphasic explanation applies to our precipitation reactions as well can be seen in the TEM data. Comparison of the morphology of the earliest crystals with that of those that emerge later shows that crystals grown by conversion of ACP are not shape-stable. Instead, a marked change in the relative proportion between crystal dimensions takes place with aging. Such alterations in crystal shape indicate an underlying phase change or, at the least, a change in the atomic configuration and/or composition on one set of growing faces (Strickland-Constable, 1968).

The octacalcium phosphate (OCP) precursor model proposed by Brown (1966) to explain the formation of nonstoichiometric apatites ($\text{Ca/P} < 1.67$) provides a possible interpretation of the sequence of events that take place during the early ripening stage of our reactions. In Brown's original proposal, the first crystals formed at physiologic pH and temperature are postulated to be two-dimensional growths of single unit-cell thick OCP. This growth pattern would account for the flaky appearance of the earliest crystals observed in the present study, as well as for their solubility approaching the thermodynamic value for OCP. However a Ca/P molar ratio of 1.40 and HPO_4^{2-} content of less than 16% (vs. 1.33 and 33% respectively, for OCP), together with crystal thicknesses less than 18 Å, suggest that some of the HPO_4^{2-} groups comprising the surface boundary of the OCP unit cell layer are absent in the earliest formed crystals.

The extreme thinness of the earliest crystals would also account for the absence of the 010 and 020 lines of OCP (at 18.7 and 9.36 Å respectively) in our X-ray patterns. There is insufficient development of lattice periodicity in this direction to generate diffraction maxima sharp enough to be detected. The observed d-spacing of 9.1 Å in the earliest crystals, on the other

hand, could be indexed as the 110 line of OCP (9.05 Å). In addition, even though the rest of the diffraction pattern of these early crystals is apatitelike, the general diffuseness of the pattern makes it impossible to exclude an OCP-like structural interpretation.

Brown's proposal of subsequent hydrolysis of the OCP-like crystals to apatite could then account for the phase change seen as responsible for the inflections in the solution data. In fact, the steady increase in the Ca/P molar ratio of the solids and the accompanying rise in solution phosphate is possible evidence that the hydrolysis to the more basic apatite phase actually begins before the inflection point is reached. The inflections in the solution data may represent that stage in the hydrolysis when not enough of the precursor phase remains to sustain the existing solubility and the solution quickly becomes subsaturated with respect to this phase as the solution readjusts to the now dominant, less soluble apatite. This surface-initiated hydrolysis is also consistent with the findings that the solution events are unaffected by stirring rate and that the crystal changes appear to occur *in situ*.

The hydrolysis step, however, does not lead directly to stoichiometric HAP. One possible explanation is the burying of part of the original OCP layer during hydrolysis. Alternatively, the low concentrations of OH^- available in solution during hydrolysis could result in OH^- deficiencies in the lattice preventing the development of more basic apatites. This latter explanation is supported by the finding that in our CO_2 -free systems extended aging (16 h at 37°) did not produce apatites with Ca/P's exceeding 1.57 despite reductions in HPO_4^{2-} to negligible levels. Direct evidence for the presence of severe OH^- deficiencies in synthetic apatites prepared at pH 7.4 was previously reported by Termine and Lundy (1973) from infrared spectral studies.

Notably, only the solution and morphologic data, and not the chemistry of the solids, revealed the existence of a post-ACP phase change. A similar situation was also observed for the original amorphous to crystalline conversion. As an example, in a pH-stat experiment conducted at 37° (procedure I), the ACP solids collected at 3 min had a Ca/P ratio of 1.42 and contained 15.4% HPO_4^{2-} whereas the first crystalline precipitate isolated 3 min later had a ratio of 1.43 and 14.7% HPO_4^{2-} . These essentially identical chemical results are in marked contrast to the dramatic solution changes observed between 3 and 6 min (see Figs. 1 and 4). In interesting contrast to these two phase changes, the final apatitic stage of the maturation process is marked mostly by changes in the chemistry of the solids. As an example (see Fig. 9), a significant increase in the Ca/P ratio of the solids between 60 and 90 min occurred (from 1.46 to 1.50), but was accompanied by very little decrease in solution calcium. This latter

finding would indicate that the increase in crystal mass during this time span from newly deposited material was negligible and, consequently, could not account for the observed increase in Ca/P ratio. Instead the data suggest that significant amounts of the earliest apatite formed by hydrolysis of the crystalline precursor had changed over into more stoichiometric material during this period, most probably through a continuous dissolution/growth process such as Ostwald ripening (Walton, 1967; Dunning, 1973).

The finding by Nancollas and Tomažič (1974), that the seeded growth of apatite from stable supersaturated solutions is preceded by the formation of an unstable OCP precursor, is in agreement with the postulate presented in this report concerning the nature of the initial crystals formed from ACP. Reinforcing this agreement was the TEM observation that the first visible growths associated with the seeds were identical in appearance and size to the crystals formed directly by ACP nucleation (Eanes, 1976). The report by Brečević and Füedi-Milhofer (1972), describing OCP as the end product of their precipitation reactions, is likewise consistent with the data given in this report. By allowing the pH of their reaction to drop to acidic values, these authors were able to stabilize what normally would be a transient intermediate phase at pH 7.4.

Calcified tissue, especially young, actively mineralizing tissue, contains ACP in addition to apatite (Termine, 1972). Although direct evidence for an ACP to apatite conversion step in vivo has yet to be firmly established, it is highly probable that some apatite crystals may form by this route. These crystals could very well undergo the same two-phase postconversion step seen in our synthetic systems.

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References

- Blumental, N.C., Betts, F., Posner, A.S.: Nucleotide stabilization of amorphous calcium phosphate. *Mat. Res. Bull.* **10**, 1055–1060 (1975)
- Boskey, A.L., Posner, A.S.: Conversion of amorphous calcium phosphate to microcrystalline hydroxyapatite. A pH-dependent, solution-mediated, solid-solid conversion. *J. Phys. Chem.* **77**, 2313–2317 (1973)
- Boskey, A.L., Posner, A.S.: Magnesium stabilization of amorphous calcium phosphate. *Mat. Res. Bull.* **9**, 907–916 (1974)
- Boskey, A.L., Posner, A.S.: Formation of hydroxyapatite at low supersaturation. *J. Phys. Chem.* **80**, 40–45 (1976)
- Brečević, L., Füedi-Milhofer, H.: Precipitation of calcium phosphates from electrolyte solutions. II. The formation and transformation of the precipitates. *Calcif. Tiss. Res.* **10**, 82–90 (1972)
- Brown, W.E.: Crystal growth of bone mineral. *Clin. Orthop.* **44**, 205–220 (1966)
- Chughtai, A., Marshall, R., Nancollas, G.H.: Complexes in calcium phosphate solutions. *J. Phys. Chem.* **72**, 208–211 (1968)
- Davies, C.W.: Ion association. London: Butterworth 1962
- Dunning, W.J.: Ripening and ageing processes in precipitates. In: Particle growth in suspensions, (Smith, A.L., ed.), pp. 3–28. London: Academic Press 1973
- Eanes, E.D.: The interaction of supersaturated calcium phosphate solutions with apatitic substrates. *Calcif. Tiss. Res.* **20**, 75–89 (1976)
- Eanes, E.D., Gillissen, I.H., Posner, A.S.: Intermediate states in the precipitation of hydroxyapatite. *Nature (Lond.)* **208**, 365–367 (1965)
- Eanes, E.D., Posner, A.S.: Kinetics and mechanism of conversion of noncrystalline calcium phosphate to crystalline hydroxyapatite. *Trans. N.Y. Acad. Sci.* **28**, 233–241 (1965)
- Eanes, E.D., Posner, A.S.: Intermediate phases in the basic solution preparation of alkaline earth phosphates. *Calcif. Tiss. Res.* **2**, 38–48 (1968)
- Eanes, E.D., Posner, A.S.: A note on the crystal growth of hydroxyapatite precipitated from aqueous solutions. *Mat. Res. Bull.* **5**, 377–384 (1970)
- Eanes, E.D., Termine, J.D., Nylen, M.U.: An electron microscopic study of the formation of amorphous calcium phosphate and its transformation to crystalline apatite. *Calcif. Tiss. Res.* **12**, 143–158 (1973)
- Fleisch, H., Russell, R.G.G., Bisaz, S., Termine, J.D., Posner, A.S.: Influence of pyrophosphate on the transformation of amorphous to crystalline calcium phosphate. *Calcif. Tiss. Res.* **2**, 49–59 (1968)
- Francis, M.D.: The inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphates. *Calcif. Tiss. Res.* **3**, 151–162 (1969)
- Gee, A., Deitz, V.R.: Pyrophosphate formation upon ignition of precipitated basic calcium phosphates. *J. Amer. chem. Soc.* **77**, 2961–2965 (1955)
- Gregory, T.M., Moreno, E.C., Brown, W.E.: Solubility of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ in the system $\text{Ca}(\text{OH})_2\text{--H}_3\text{PO}_4\text{--H}_2\text{O}$ at 5, 15, 25, 37.5° C. *J. Res. Nat. Bur. Stand.* **74a**, 461–475 (1970)
- Madsen, H.E.L.: Ionic concentrations in calcium phosphate solutions. I. Solutions saturated with respect to brushite or tetra-calcium monohydrogen phosphate at 37° C. *Acta chem. scand.* **24**, 1671–1676 (1970)
- McDowell, H., Wallace, B.M., Brown, W.E.: The solubilities of hydroxyapatite at 5, 15, 25 and 37°C. Program and abstracts. 47th General Meeting IADR, Abstract No. 340, 1969
- Meyer, J.L., Eick, J.D., Nancollas, G.H., Johnson, L.N.: A scanning electron microscopic study of the growth of hydroxyapatite crystals. *Calcif. Tiss. Res.* **10**, 91–102 (1972)
- Meyer, J.L., McCall, J.T., Smith, L.H.: Inhibition of calcium phosphate crystallization by nucleoside phosphates. *Calcif. Tiss. Res.* **15**, 287–293 (1974)
- Meyer, J.L., Nancollas, G.H.: The influence of multidentate organic phosphonates on the crystal growth of hydroxyapatite. *Calcif. Tiss. Res.* **13**, 295–303 (1973)
- Moreno, E.C., Patel, J.M., Gregory, T.M., Brown, W.E.: Solubility of Whitlockite. $\beta\text{-Ca}_3(\text{PO}_4)_2$. Program and abstracts. 48th General Meeting IADR, Abstract No. 183, 1970
- Murphy, J., Riley, J.P.: A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta* **27**, 31–36 (1962)

- Nancollas, G.H., Mohan, M.S.: The growth of hydroxyapatite crystals. *Arch. oral Biol.* **15**, 731–745 (1970)
- Nancollas, G.H., Tomažič, B.: Growth of calcium phosphate on hydroxyapatite crystals. Effect of supersaturation and ionic medium. *J. Phys. Chem.* **78**, 2218–2225 (1974)
- Robertson, W.G.: Factors affecting the precipitation of calcium phosphate in vitro. *Calcif. Tiss. Res.* **11**, 311–322 (1973)
- Strickland-Constable, R.F.: Kinetics and mechanism of crystallization. London: Academic Press 1968
- Termine, J.D.: Mineral chemistry and skeletal biology. *Clin Orthop.* **85**, 207–241 (1972)
- Termine, J.D., Conn, K.M.: Inhibition of apatite formation by phosphorylated metabolites and macromolecules. *Calcif. Tiss. Res.* **22**, 149–157 (1976)
- Termine, J.D., Lundy, D.R.: Hydroxide and carbonate in rat bone mineral and its synthetic analogues. *Calcif. Tiss. Res.* **13**, 73–82 (1973)
- Termine, J.D., Peckauskas, R.A., Posner, A.S.: Calcium phosphate formation in vitro. II. Effect of environment on amorphous-crystalline transformation. *Arch. Biochem. Biophys.* **140**, 318–325 (1970)
- Tomazič, B., Nancollas, G.H.: The seeded growth of calcium phosphates. Surface characterization and the effect of seed material. *J. Colloid Interface Sci.* **50**, 451–461 (1975)
- Tomazič, B., Tomson, M., Nancollas, G.H.: The growth of calcium phosphates on natural enamel. *Calcif. Tiss. Res.* **19**, 263–271 (1976)
- Walton, A.G.: The formation and properties of precipitates. New York: Interscience 1967
- Walton, A.G., Bodin, W.J., Füredi, H., Schwartz, A.: Nucleation of calcium phosphate from solution. *Canad. J. Chem.* **45**, 2695–2701 (1967)

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